REMARKS

The Invention

The application discloses methods and kits for isolating nucleic acids from a sample. The methods use a solid support that includes an organic polymer, and the nucleic acid is bound to the support in the presence of a detergent and in the absence of any chaotropic agent.

The Office Action

Claims 1-4, 9-11, 13, 16-18, 20 and 22-24 have been rejected as being anticipated under 35 USC 102(e) by Cros et al., US Patent No. 5, 510, 084 (referred to herein as "the Cros patent"). The Examiner has also rejected claims 1-24 under 35 U.S.C. §103(a) over the Cros patent, in combination with the 1994 Pharmacia Technology Products Catalog ("the Pharmacia catalog"). Claims 5 and 24 have been rejected under 35 U.S.C. §112, second paragraph. The Examiner has withdrawn the previous rejections of claims 1, 2 and 14, and 13 and 19-21 for anticipation and indefiniteness, respectively.

Pending Claims

Claims 5 and 24 have been amended, and claims 25-26 have been added. Upon entry of this amendment claims 1-26 will be pending in the application. No new subject matter has been added.

Claim 5 has been amended to specify the additional step of disrupting or lysing structural components or cells in the sample and the timing of such step, i.e., prior to the contacting step. Support for this claim amendment can be found, e.g., in the last paragraph of page 6 of the specification.

Claim 24 has been amended to recite the biochemical manipulation encompassed by this claim, i.e., detection, hybridization, amplification or quantification of the bound nucleic acid. Support for this claim amendment can be found, e.g., on page 1, lines 9-10 and 20-22; page 12, lines 10-17; page 13, lines 20-22; and in Examples 6 and 10 of the specification.

New claims 25-26 have been added. Support for these new claims can be found, e.g., on page 5, lines 21-22 and in the last paragraph of page 6 of the specification.

In view of the foregoing claim amendments and the following remarks, it is respectfully submitted that the application is in condition for allowance.

Rejection of Claims 5 and 24 under 35 U.S.C. 112, second paragraph

On page 2, lines 11-30 of the outstanding Office Action, the Examiner rejects claims 5 and 24 under 35 U.S.C. 112, second paragraph, "as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention."

With respect to claim 5, the Examiner states that the meaning of the phrase "additional steps" is unclear.

This rejection has been met by amending this claim to delete the language objected to by the Examiner, and by specifying the additional step of disrupting or lysing structural components or cells in the sample and the timing of such step, i.e., prior to the contacting step.

Claim 24 has been rejected because of its recitation of the term "biochemical manipulation," which according to the Examiner "lacks specificity and is indefinite with regards to the number of biochemical techniques available to one of skill in the art."

This rejection has been met by amending claim 24 to recite the biochemical manipulations encompassed by this claim, i.e., detection, hybridization, amplification or quantification of the bound nucleic acid.

In view of the foregoing, Applicants respectfully request that the rejection of claims 5 and 24 under 35 U.S.C §112, second paragraph, be withdrawn.

Rejection of Claims 1-4, 9-11, 13, 16-18, 20, 22-24 under 35 U.S.C. 102(e)

On page 5 of the outstanding Office Action, the Examiner rejects claims 1-4, 9-11, 13, 16-18, 20, and 22-24 under 35 U.S.C. 102(e) as being anticipated by Cros et al., U.S. Patent No. 5,510,084. According to the Examiner:

> Cros anticipates these claims as it teaches the use of an organic polymeric support in the separation and isolation of nucleic acids of either DNA or RNA. Cros et al. teach the use of natural or synthetic materials which may or may not be chemically modified as solid supports such as nylon or polyacrylate. Cros et al. teach polymers such as polydivinylbenzene, polystyrene, polypropylenes, polyethylene and copolymers thereof

as preferred supports for the immobilization of nucleic acids in diagnostic tests, affinity chromatography and in separation processes (col. 4, line 17- col. 5, line 4). Cros et al. also teaches the use of detergents such as alkaline sodium phosphate in the isolation process (see Example 12).

Applicants respectfully traverse the above-quoted rejection.

The rejected claims are directed to methods and kits for isolating nucleic acids (e.g., DNA, RNA) from a sample. The method requires contacting the sample with a solid support in the presence of a detergent and the absence of a chaotropic agent. Thus, the rejected claims require the binding/contacting of the nucleic acid in the sample to the solid support to occur in the presence of a detergent and the absence of a chaotropic agent.

The Cros patent fails to disclose, expressly or inherently, a process for isolating a nucleic acid where the binding of the nucleic acid to a solid support occurs in the presence of a detergent and the absence of a chaotropic agent, as required by the rejected claims. The Cros patent discloses a process for non-covalently immobilizing, on a solid support, a nucleic acid fragment (preferably, a fragment containing fewer than 100 nucleotides) modified to be coupled to a ligand. The covalently-bound ligand moiety of the modified nucleic acid fragment immobilizes the nucleic acid fragment to the solid support. This reference further describes the use of the immobilized fragment as a "capture oligonucleotide" to recognize and bind a target sequence having a specific predefined sequence.

Example 12, which is pointed out by the Examiner as allegedly describing "the use of detergents such as alkaline sodium phosphate in the isolation process" does not mention the use of a detergent in the nucleic acid binding/contacting step. This Example simply describes the use of four sodium phosphate buffers at different pH's to immobilize the capture oligonucleotide to the solid support. No detergent is included in the Cros patent's binding step. Alkaline sodium phosphate is a buffer solution, not a detergent. A detergent must comprise a surface active ingredient. The following is a definition taken from The Comiti International de Dirivis Tensio Actifs:

Surface Active Agent: Chemical compound which, when dissolved or dispersed in a liquid is preferentially absorbed at an interface, giving rise to a number of physico-chemical or chemical properties of practical interest. The molecule of the compound includes at least one group with an affinity for markedly polar surfaces, ensuring in most cases solubilization in water, and a group which has little affinity for water.

The sodium phosphate used as a buffer in Example 12 does not satisfy the above-described requirements of a detergent, e.g., a group with little affinity for water. For example, sodium dodecyl sulphate (SDS), which is listed in the last paragraph on page 7 of the specification as an example of a detergent that can be used in the methods of the present invention, has a dodecyl group that has little affinity for water and a sulphate group that has affinity for polar surfaces. Sodium phosphate does not have these properties since it lacks the lipophilic group.

The Cros patent does disclose the use of detergent- (i.e., Tween-) containing solutions in the washing steps to remove unbound fragments; however, these washes occur after the binding step has taken place. Nowhere does Cros suggest any reason to use a detergent in the initial step when the sample is first contacted with a solid support and a soluble nucleic acid in the sample becomes bound to the support, as required by the present claims.

Furthermore, the rejected claims require that the soluble nucleic acid in the sample "is bound" to the solid support. Thus, the claims require direct binding of the nucleic acid to the soluble support. In contrast, in the method of the Cros patent, the nucleic acid molecules are isolated by binding to complementary oligonucleotides that are covalently attached to ligands that are largely proteinaceous and/or hydrophobic in nature. These ligands bind non-covalently to the solid support primarily via hydrophobic and polar interactions (see column 24, last paragraph of the Cros patent). Therefore, the binding of the nucleic acids to the solid support in the Cros methods is indirect, as opposed to the direct interaction to the support required by the present claims.

Since the rejected claims require direct binding of the nucleic acid in the sample to the support, and require this binding to occur in the presence of the detergent, the Cros patent fails to disclose, expressly or inherently, each and every element required by the rejected claims.

Accordingly, claims 1-4, 9-11, 13, 16-18, 20 and 22-24 are not anticipated by this reference. A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference [See MPEP 2131, quoting *Verdegaal Bros v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987)].

Reconsideration and withdrawal of this rejection of claims 1-4, 9-11, 13, 16-18, 20 and 22-24 is respectfully requested.

Rejection of Claims 1-24 under 35 U.S.C. 103(a)

On pages 4-5 of the outstanding Office Action, the Examiner rejects claims 1-24 under 35 U.S.C. 103(a) as being obvious over Cros et al., U.S. Patent No. 5,510,084, described above, and the 1994 Pharmacia Biotechnology Products Catalog ("the Pharmacia catalog") already of record. To support this rejection, the Examiner repeats previous arguments that:

> One of skill in the art would clearly recognize the common use of detergents within the art of purification of nucleic acids; moreover the Pharmacia product could be used in the absence of a chaotropic agent, barring evidence to the contrary, which has not been presented by applicant; moreover, detergents listed in the specification (p.8) such as Tris, Bicine, Tricine and phosphate buffers have been routinely used in this art for the preparation, isolation or separation of nucleic acids (see Cros et al. cited below). Given this, one of skill in the art would clearly recognize the common use of detergents within the art of purification of nucleic acids.

The Examiner cites the Cros patent "to show the obviousness of the use of solid supports and non-chaotropic buffers in the isolation or separation of nucleic acids."

The pending claims are directed to methods for isolating a nucleic acid (e.g., DNA, RNA) from a sample by contacting the sample with a solid support in the presence of a detergent and the absence of a chaotropic agent. Kits for carrying the aforesaid methods are also disclosed.

The cited references, alone or in combination, fail to render obvious the claimed invention. As discussed in more detail below, even if these references are combined as suggested by the Examiner, their combination does not disclose or suggest the claimed invention, i.e., a method for isolating a nucleic acid from a sample where the contacting of the nucleic acid in the sample to the solid support occurs (i) in the presence of a detergent and (ii) in the absence

of a chaotropic agent, and where the nucleic acid becomes bound to the solid support itself (as opposed to being bound to a capture probe or a ligand that is fixed to the solid support).

The cited references are discussed individually and in combination below.

The Pharmacia catalog discloses an oligo(dT)-cellulose affinity column for mRNA isolation. This column is used for the sequence-specific separation of poly(A)-containing mRNA from a sample. The cited catalog fails to disclose or suggest that the claimed method could or should be carried out (i) in the presence of a detergent and (ii) in the absence of a chaotropic agent, or that the binding is directly to the solid support.

The secondary reference of Cros fails to make up for the deficiencies of the Pharmacia catalog. As described above, the Cros patent discloses a process for non-covalently and indirectly immobilizing, on a solid support, a nucleic acid fragment modified to be coupled to a ligand. The covalently-bound ligand moiety of the modified nucleic acid fragment immobilizes the nucleic acid fragment indirectly on the solid support. This reference further describes the use of the immobilized fragment as a "capture oligonucleotide" to recognize and bind a target sequence having a specific predefined sequence. The Cros patent discloses isolation of target molecules from a purified sample, i.e., a sample that has been already purified to isolate the total nucleic acid material.

The Examiner cites to Cros to support the statement that "detergents listed in the specification (p. 8) such as Tris, Bicine, Tricine and phosphate buffers have been routinely used in this art for the preparation, isolation or separation of nucleic acids." Contrary to the Examiner's belief, the Cros patent does not mention the use of a detergent in the nucleic acid isolation/contacting step. The "Tris, Bicine, Tricine and phosphate buffers" listed by the Examiner are buffer solutions, not detergents. The differences between detergents and buffers are described in response to the Examiner's rejection of the claims under 102(e) above. Since there is no suggestion in either of the cited references to utilize a detergent in the contacting step, the Examiner has failed to make out a *prima facie* case of obviousness, and the rejection should be withdrawn on this basis alone.

Furthermore, there is no suggestion in either reference that the contacting step be carried out in the absence of a chaotropic agent, as required by the present claims. The Examiner makes reference to the fact that there is "no evidence that one of skill in the art could not use the

claimed Pharmacia invention in the absence of chaotropic agents" and relies on the Cros disclosure as evidence that non-chaotropic buffers may be used in the isolation methods. The issue is not whether one of ordinary skill in the art could use the Pharmacia product with non-chaotropic buffers, but whether one would have been motivated do so.

Applicants submit that the Pharmacia products were routinely used with chaotropes, as evidenced by Pharmacia patent, US 5,459,253, which describes the use of oligo(dT) solid supports, and Quick Prep instructions (relevant copies of which are submitted herewith as Appendices A and B). These materials explicitly require the use of chaotropes as imperative in the procedure (see, e.g., the summary of the invention, column 2 of Patent '253). The chaotrope is used to allow purification directly from a cell (and to inhibit RNAses). The only circumstances under which the chaotrope might not be used is if purification from a cell was not being performed and RNA was not being isolated. Therefore, the products described in the Pharmacia catalog (which are used to isolate RNA) would be used as described by the Pharmacia '253 patent, i.e., with chaotrope-containing buffers, in contrast to the claimed methods. The Examiner has not identified any evidence to the contrary.

Finally, the methods disclosed in the Pharmacia catalog and the Cros patent differ drastically from the claimed invention in that the target nucleic acid of each reference is not bound directly to the solid support. In each case, the target nucleic acid binds by hybridization to the capture probe that in turn is non-covalently bound to the solid support. Thus, neither reference, nor the two in combination, can be said to disclose a method for binding a nucleic acid directly to a solid support, much less doing so in the presence of a detergent and absence of a chaotropic agent, or any reason to seek a way of doing so.

A rejection for obviousness requires that the prior art teach or suggest all of the claim limitations. MPEP 2142. In the present case, the rejection for obviousness is flawed because the cited references fail to disclose or suggest that the binding of the nucleic acid could or should occur (i) in the presence of a detergent and (ii) in the absence of a chaotropic agent, or that the nucleic acid should be directly bound to the solid support.

Applicants respectfully submit that newly added claim 26 is also inventive over the cited art. Claim 26 is directed to a method for isolating a nucleic acid from a sample by contacting the sample and a solid support in the presence of a detergent and the absence of a chaotropic agent,

whereby the soluble nucleic acid in the sample is bound to the support in a sequence-independent manner. This claim is clearly distinct from the disclosures of the Pharmacia catalog and the Cros patent, as discussed below.

In the Pharmacia catalog method, the cellulose resin linked to oligo(dT) or oligo(dA) is used to recognize and bind target sequences having specific predefined sequences, e.g., mRNA with poly(A) tails or oligonucleotides that have a sequence complementary to dT or dA. The Examiner has countered Applicants' previous arguments by indicating that the Pharmacia catalog is also concerned with fractionation of oligonucleotides and therefore uses to isolate molecules other than poly(A)-containing RNAs are also contemplated. The Examiner's comments in this regard are also respectfully traversed.

The catalog provides that the method disclosed is the method of choice for mRNA isolation and also is used for purification of nucleic acid enzymes, binding of steroid receptors and fractionation of oligonucleotides." The catalog states that the Selection Guide may be used to determine the most appropriate adsorbent for the application. A copy of the corresponding 1992 catalog describes the application of four oligo(dT)/(dA) solid supports produced by Pharmacia. The relevant pages are submitted herewith as Appendix C. The preamble of the 1992 catalog (Appendix C) has the same language as the cited Pharmacia catalog and reference is made to fractionation of oligonucleotides. However, all of the applications provided in the Selection Guide rely on sequence-specific binding, since the "ligand" is a poly(T) or poly(A) sequence. The applications stated in the Selection Guide are to isolate RNA containing poly(A) or poly(U) sequences. Indeed, it is stated as one of the features that the oligodeoxynucleotide celluloses are highly specific for binding poly(A) and isolate poly(A)-containing mRNA from 50-100 fold quantities of rRNA. The fractionation description concerns methods involving binding to specific polyA or T sequences. This may be, for example, to isolate the total mRNAs which could then be separated (i.e., by fractionation) on the basis of different lengths. Thus, the Pharmacia catalog only describes methods involving sequence-dependent binding.

The Cros method also requires sequence-specific binding. This method employs a ligand to attach the capture nucleic acid molecules to the solid support. Examples 7 and 12 of the Cros patent describes a "sandwich" technique in which an immobilized fragment is used as a "capture oligonucleotide" to recognize and bind a target sequence having a specific predefined sequence.

In fact, the Cros patent provides an improved process for hybridizing target nucleic acid sequences to capture probes immobilized on a solid support because it maximizes the nucleotides exposed for hybridization. The covalently-bound ligand moiety of the modified nucleic acid fragment serves to immobilize the nucleic acid fragment on the solid support, thus maximizing the nucleotides available for hybridization. The target nucleic acid is indirectly attached to the solid support through sequence-specific binding, whereas in the present invention the binding of the nucleic acid to the solid support is direct.

Therefore, the two references cited by the Examiner describe sequence-specific binding indirectly to the solid support, i.e., binding to oligo(dT) or oligo(dA), or to a particular immobilized oligonucleotide, respectively. The oligodeoxynucleotide celluloses of Pharmacia are used for affinity isolation by virtue of specific interactions between complementary bases of the target and capture entities. In contrast, in the invention of claim 26, binding is sequence-independent, and nucleic acid molecules bind non-specifically to the solid support (see page 5, lines 21-22, of the specification). The Pharmacia catalog and the Cros patent are not concerned with, nor suggestive of, the claimed method in which sequence-independent binding occurs.

Thus, the invention of claim 26 is concerned with a new approach for isolating nucleic acid molecules, which would not have been obvious in view of the cited art. The cited references fail to disclose or suggest modifying the Pharmacia or Cros sequence-dependent method by including detergent, avoiding use of a chaotropic agent, and binding in a sequence-independent manner. Nor does either reference suggest a reason that sequence-independent binding might be desirable.

The present invention allows, for the first time, the removal of chaotropes from the nucleic acid isolation method even when using samples which are not isolated nucleic acid material. Effective binding of the nucleic acid material is achieved in the presence of a detergent. Rapid and direct isolation of nucleic acid material is provided by the present invention, which is not reliant on any sequence information about the target. Instead, the total nucleic acid material is isolated, from which, thereafter, the techniques of Pharmacia and/or Cros could be used to isolate particular target sequences. Thus, the methods of the present invention provide an alternative to known methods of precipitation and extraction of bulk nucleic acid material, and were not foreshadowed by the prior art.

In view of the foregoing, no *prima facie* case of obviousness has been established for the claimed methods and kits. Applicants respectfully request that the rejection of the pending claims under U.S.C. §103 as being unpatentable over the Cros et al. patent and the Pharmacia catalog be withdrawn.

SUMMARY

The present claims are in condition for allowance.

Amendment of these claims should not be construed as an acquiescence to the Examiner's rejection. These amendments are being made solely for the purpose of expediting prosecution of the above-identified application. Applicant reserves the right to pursue the claims in this application or another application.

If a telephone conversation with Applicant's Attorney would expedite the prosecution of the above-identified application, the Examiner is urged to call Applicant's Attorney at (617) 542-5070.

A petition for an extension of time and check for the required fee are being filed concurrently herewith. A check to cover excess claim fees is also submitted herewith. Please apply any additional charges or credits to Deposit Account No. 06-1050, referencing attorney docket number 08269-003001.

Respectfully submitted,

Date: April 10, 2001

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Version with markings to show changes made

In the claims:

Claims 5 and 24 have been amended as follows:

5. (First time amended) A method as claimed in claim 1, further comprising disrupting or lysing structural components or cells in the sample prior to the contacting step. [one or more additional steps to disrupt structural components in the sample or to achieve lysis of cells in the sample.]

24. (First time amended) A method as claimed in claim 1, the method further comprising the step of detecting, hybridizing, amplifying or quantifying [subjecting] the bound nucleic acid [to further biochemical manipulation] after the separating step.

Appendix B

READ ME FIRST

- This is an electronic version, produced in Adobe Acrobat, of the instructions supplied with the product.
- On most monitors, use of the file is simplest at 115-150% magnification in Acrobat. This magnification should make the print size easy to read and permit orientation through the instructions.
- The page numbering of the Acrobat file does not match the page numbering of the instruction booklet, since two instruction booklet pages have been assembled onto one Acrobat page. Therefore, references to page numbers within the instructions are referring to the page numbers within the instructions and not to the numbers of the Acrobat pages.

PHARMACIA BIOTECH

QuickPrep® mRNA Purification Kit INSTRUCTIONS

QuickPrep® mRNA Purification Kit* is designed for the direct isolation of polyadenylated RNA from eukaryotic cells or tissues, bypassing the need for intermediate purification of total RNA. Each purification can be performed in approximately 1 hour starting from as little as one cell or as much as 0.5 g of tissue. mRNA isolated with the kit can be used in numerous applications, such as cDNA synthesis, Northern analysis, *in vitro* translation and PCR (1). Sufficient reagents are provided for four mRNA purifications.

*U.S. Patent No. 5,459,253 has been issued to Pharmacia P-L Biochemicals Inc. for coupled extraction and affinity purification of mRNA from eukaryotic cells.

XY-026-00-09
Rev. 5

Pharmacia Biotech

CONTENTS

COMPONENTS

All components (except those for the precipitation step) are packaged in such a way that each mRNA purification can be performed with previously unopened materials.

Oligo(dT)-Cellulose Spun Columns (4):	Oligo(dT)-cellulose suspended in a storage buffer containing 0.15% Kathon CG/ICP Biocide*.
Extraction Buffer (4 vials):	A buffered aqueous solution containing guanidinium thiocyanate and N-lauroyl sarcosine.
High-Salt Buffer (4 vials):	10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 M NaCl.
Low-Salt Buffer (4 vials):	10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M NaCl.
Elution Buffer (4 vials):	10 mM Tris-HCl (pH 7.5), 1 mM EDTA.
Glycogen Solution (2 vials):	5-10 mg/ml glycogen in DEPC-treated water.
K Acetate Solution (2 vials):	2.5 M potassium acetate (pH 5.0).
Sample Buffer (2 vials):	10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 3.0 M NaCl.

* Trademark of Rohm and Haas Company

Additional reagents and the equipment required are listed in Appendix 1, page 17.

OVERVIEW

QuickPrep mRNA Purification Kit is designed for the rapid isolation of mRNA from eukaryotic cells or tissues without the need for intermediate purification of total RNA. Each purification can be completed in as little as 1 hour depending on the purity of mRNA desired, the amount of starting material, and the mRNA content of this material. The kit is especially well suited for the isolation of mRNA from cells or tissues which are available in only limited quantities, because it eliminates losses associated with intermediate purification of total RNA. We have isolated mRNA from as little as one cell and as much as 0.5 g of tissue. For studies in which a larger amount of starting material is available and large amounts of pure mRNA are required, we suggest the use of the RNA Extraction Kit (27-9270-01) in conjunction with the mRNA Purification Kit (27-9258-01, -02).

QuickPrep mRNA Purification Kit combines the disruptive and protective properties of guanidinium thiocyanate (GTC, ref. 2 and 3) with the speed and selectivity of oligo(dT)-cellulose chromatography in a spun-column format pioneered by Pharmacia (4). The protocol is outlined on page 6 and briefly described below.

Initially, the tissue is extracted by homogenization in a buffered solution containing a high concentration of GTC. This ensures the rapid inactivation of endogenous RNase activity and the complete dissociation of cellular components from the mRNA.

The extract is then diluted three-fold with Elution Buffer, to reduce the GTC concentration to a level which we have selected very carefully: low enough to allow efficient hydrogen-bonding between poly(A) tracts on mRNA molecules and oligo(dT) attached to cellulose, but high enough to maintain complete inhibition of RNases. As an added benefit, the three-

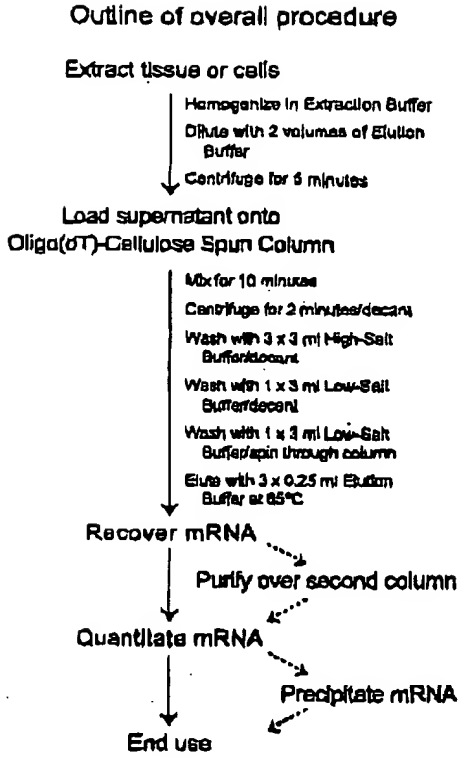
fold dilution causes a number of proteins to precipitate, allowing them to be removed easily by centrifugation.

After a brief second homogenization, the extract is clarified by centrifugation, the supernatant is poured into an Oligo(dT)-Cellulose Spun Column, and the polyadenylated fraction is allowed to bind over a short period of time with gentle mixing. The column is subjected to a low-speed centrifugation, and the liquid containing the non-bound material is decanted. The matrix is batch-washed sequentially with High-Salt and then Low-Salt Buffer. Finally, the sample is eluted from the matrix with prewarmed Elution Buffer.

Each Spun Column has the capacity to bind up to 25 µg of polyadenylated RNA. Purity is dependent on the number of cells extracted. If fewer than 10^7 cells are extracted, the mRNA isolated will have a purity of greater than 90%; with progressively larger tissue samples, the purity may gradually drop to ~ 50% (or in rare instances, even lower).

The RNA isolated with the kit is essentially free of DNA and protein contamination. It should be of sufficient purity for most end uses. If further purification of the sample is desired, it may be subjected to a second spun-column purification as described in Appendix 2. Note, however, that the use of a second column will consume a second set of reagents, thus decreasing the total number of purifications possible with the kit. In any case, the use of a second column may not be advisable when purifying mRNA from small amounts of cells, because it may reduce the final yield below useful levels for some applications.

The kit contains both potassium acetate and glycogen solutions for precipitation of mRNA in cases where it is not sufficiently concentrated for use directly in the selected application.



PROTOCOL

Introduction

Below are several points which should be reviewed prior to beginning the procedures.

RNase-Free Conditions

The most important consideration in any purification of RNA is protection of the sample from contamination with ribonucleases (RNase). The reagents provided with QuickPrep mRNA Purification Kit are tested for RNase contamination prior to shipment. Any other plastic- or glassware which may come into contact with the sample should be autoclaved or otherwise treated to prevent RNase contamination (5). Fresh gloves should be worn during the purification, both to protect the researcher from contact with solutions and to protect the RNA from nucleases present on the skin. Protective eyewear should be worn at all times.

Spun-Column Chromatography

A centrifuge with a swinging-bucket rotor capable of accommodating a 15 ml centrifuge tube with a spun column inserted into it should be used. For consistent results, it is important to maintain the correct time and speed of centrifugation when using the Oligo(dT)-Cellulose Spun Columns.

- Timer: A digital timer should be used to ensure consistent run times. Variation in the time of centrifugation will cause discrepancies between the volume of solution applied and the volume of sample eluted.

- Speed: For a force of 350 x g, calculate the required speed using the following formula: RCF = 350 = (1.12) (r) (rpm/1000)^2, where RCF = relative centrifugal force; r = radius in mm measured from center of spindle to bottom of rotor bucket; and rpm = revolutions per minute. This equation resolves to:

$$rpm = \frac{1.77 \times 10^4}{\sqrt{r}}$$

  For example, with a rotor having a radius of 170 mm, the appropriate speed would be 1,360 rpm. We have found it useful to mark the position on the speed control of the centrifuge which will yield the correct RCF.

The buckets, column and counterweight should be balanced prior to centrifugation. This is especially important during sample elution.

Occasionally, a very small amount of resin may leak from the column during the draining step and centrifugations. This leakage is minimal and should not interfere with the performance of the column.

The columns have a finite capacity and can be overloaded. Overloading the columns will result in a decrease of the A_{260}/A_{280} ratio of the final product and thus the purity of the mRNA isolated.

The columns may become slippery if Extraction Buffer is spilled on the outside surface, so exercise caution in handling them.

Essential Preliminaries

- Procedures A and B must be performed without pause, from disruption of cells or tissue to elution of the poly(A)$^+$ RNA.
- Make sure that all materials are ready before starting the procedure (see also Appendix 1). The Extraction Buffer should be warmed to room temperature, with any crystallized material completely dissolved, before use (see Procedure A).
- Work at room temperature except where specifically directed otherwise.

The instructions which follow are for a single column purification. If a second column purification step is desired, refer to Appendix 2.

In the text which follows, materials provided as components of the kit appear in boldface type.

Caution:

- Extraction Buffer and K Acetate Solution are irritants and should be handled with care.

Procedure A: Extraction of Sample

> During extraction, disrupted cells may appear to "clump together", or the homogenate may become quite viscous. Neither of these phenomena will affect the outcome of the extraction. Continue the procedure uninterrupted.

- Approximately 20-30 minutes before the tissue or cell sample will be ready for extraction, remove the Extraction Buffer from storage at 4°C and place it at 37°C. Shake the bottle occasionally, until all the crystalline material is dissolved. Cool to room temperature. *Note:* If the crystalline material persists, place the bottle at 55°C and shake occasionally. If it is difficult to get the final crystals into solution, simply allow the crystalline material to settle and pipette the solution away from the crystals. This will not result in any deleterious effects on buffer performance.

If working with cultured cells, proceed as described on page 11. Otherwise, proceed as described below.

For Extraction of Tissue (up to 0.5 g)

- Place the tissue in a chilled homogenizer (either manual or mechanical) and add 1.5 ml of Extraction Buffer.
- Homogenize the tissue until it is a uniform suspension. Avoid the generation of excess heat or foam.
- To dilute the sample, add 3 ml of Elution Buffer to the extract and mix thoroughly. Homogenize briefly, then transfer the homogenate

into a sterile polypropylene centrifuge tube. Place the remaining Elution Buffer at 65°C until needed in Procedure B.

- After assuring that each tube is counter-balanced, centrifuge the diluted extract at approximately 12,000 x g (e.g. 10,000 rpm in a Beckman JA 20 rotor) for 5-10 minutes at room temperature., then proceed to Procedure B.

For Extraction of Cultured Cells (up to 5 x 10^7 cells)

- *To extract cells grown as a monolayer:* Drain the culture medium from the cells in one 75 cm^2 flask, then add 1.5 ml of Extraction Buffer directly onto the cells. Swirl the buffer over the monolayer to assure complete cell lysis. If desired, this suspension can be transferred onto an additional monolayer to lyse these cells.

  Alternately, first treat the cells with trypsin before extraction: This should be the method chosen if you plan to pool cells from several plates or flasks. Using standard procedures, treat the cells with trypsin to detach them, centrifuge to pellet them, and resuspend them in phosphate-buffered saline or similar solution. If desired, count the cells by hemocytometry and trypan blue exclusion (6). Pellet the cells by centrifugation, and discard the supernatant. Add 1.5 ml of Extraction Buffer.

  To extract cells grown in suspension: If desired, count the cells by hemocytometry and trypan blue exclusion (6). Pellet the cells by centrifugation, and decant and discard the supernatant. Add 1.5 ml of Extraction Buffer to the pelleted cells.
- To assure a homogeneous extract, disrupt the cells using a homogenizer or pass the extract through a 21-gauge needle attached to a syringe.

- To dilute the sample, add 3 ml of Elution Buffer to the extract and mix thoroughly. Homogenize briefly, then transfer the homogenate into a sterile polypropylene centrifuge tube. Place the remaining Elution Buffer at 65°C until needed in Procedure B.
- After assuring that each tube is counter-balanced, centrifuge the diluted extract at approximately 12,000 x g (e.g. 10,000 rpm in a Beckman JA 20 rotor) for 5-10 minutes at room temperature.
- Proceed to Procedure B.

Procedure B: Isolation of mRNA

- Invert an Oligo(dT)-Cellulose Spun Column several times to resuspend the matrix.
- Remove both top and bottom closures, place the column in a 15 ml centrifuge tube and balance against a counterweight. Centrifuge at 350 x g for 2 min.
- Remove the column from the centrifuge tube and discard the liquid in the tube. Replace the bottom closure on the drained column and place it upright in a rack.
- Using a sterile pipette, transfer 4 ml of the supernatant from the final step in Procedure A onto the surface of the resin of the Oligo(dT)-Cellulose Spun Column. Avoid disturbing the pelleted cellular material during this transfer.
- Replace the top closure of the column, and invert the column several times to resuspend the resin.
- Gently mix for 10 minutes by inverting the column manually or by placing it on a rocking table or similar device.
- Leaving both closures securely on, place the column in a 15 ml centrifuge tube and balance against a counterweight. Centrifuge at 350 x g for 2 minutes to separate the resin from the suspension.
- Remove the top closure, decant the supernatant and discard it. Avoid disturbing the resin.
- Apply 3 ml of High-Salt Buffer to the top of the resin and replace the top closure of the column. Resuspend the matrix by gentle mixing. This may require you to tap the bottom of the column several times. Place the column in a 15 ml tube and balance against a counterweight.

Centrifuge at 350 x g for 2 minutes. Remove the top closure, decant the supernatant and discard it.

- Repeat the wash using High-Salt Buffer two more times, exactly as described in the step above.
- Apply 3 ml of Low-Salt Buffer to the column and replace the top closure. Resuspend the matrix by gentle mixing. This may require you to tap the bottom of the column several times. Place the column in a 15 ml centrifuge tube and balance against a counterweight. Centrifuge at 350 x g for 2 minutes. Remove the top closure, decant the supernatant and discard it.
- Remove the bottom closure. Apply 3 ml of Low-Salt Buffer to the top of the resin. Balance against a counterweight. Centrifuge at 350 x g for 2 minutes.
- Place a sterile 1.5 ml screw-top microcentrifuge tube inside a 15 ml centrifuge tube. Place the column inside the 15 ml centrifuge tube in such a way that the tip of the column is inside the opening of the screw-top microcentrifuge tube (the "collection tube").
- Elute the bound poly(A)$^+$ RNA as follows, using three washes with Elution Buffer *prewarmed* to 65°C. For each wash, pipette 0.25 ml of buffer onto the top of the column; balance the column (in its collection tube) against a counterweight; then centrifuge at 350 x g for 2 minutes. Do not change the collection tube between washes, so that the entire 0.75 ml eluate is collected in the same sterile tube.
- Remove the screw-top microcentrifuge tube from the 15 ml centrifuge tube using clean (flamed) forceps. Place the collected sample and remaining Elution Buffer on ice. Proceed to Appendix 2 if a second column purification of the sample is desired.

Procedure C: Quantitation and Precipitation of mRNA

The concentration of RNA in the final eluate can be estimated by ethidium bromide fluorescence (7) or determined by spectrophotometry, as described below. Note, however, that this spectrophotometric procedure will consume one-third of the sample.

- To protect the RNA sample from degradation, pretreat the 0.5 ml cuvettes as described in Appendix 1.
- Place 0.25 ml of the column eluate in a clean microcentrifuge tube and add an equal volume of Elution Buffer. Read the absorbance of the diluted sample at 260 nm in a spectrophotometer blanked against Elution Buffer. The absorbance reading at 260 nm must be between 0.05 and 2.0 to reflect the RNA concentration accurately. If the absorbance at 260 nm is above 2, the sample should be diluted further to determine the RNA concentration. Do not return the diluted sample to the original eluate.
- Knowing that for RNA, an absorbance of 1 at 260 nm is 40 µg/ml, calculate the concentration of the RNA present in the eluate ([RNA]) using the formula:

  $[RNA] = A_{260} \times D \times 40\ \mu g/ml$

  where D = final dilution factor (in the simplest case above, this would be 2).

If the absorbance of the diluted sample is greater than or equal to 0.5, the sample may be used directly for cDNA synthesis utilizing the cDNA Synthesis Kit (27-9260-01), TimeSaver® cDNA Synthesis Kit (27-9262-01), First-Strand cDNA Synthesis Kit (27-9261-01), or Ready-To-Go™

T-Primed First-Strand Kit (27-9263-01). If the absorbance is less than 0.5, the sample must be precipitated and redissolved at a higher concentration to ensure efficient cDNA synthesis utilizing either of these kits.

- To precipitate the isolated mRNA, add 50 µl of K Acetate Solution and 10 µl of Glycogen Solution to the remaining 0.5 ml of sample. Add 1 ml of 95% ethanol (chilled to -20°C) and place the sample at -20°C for a minimum of 30 minutes. If the volume of the RNA to be precipitated is more that 0.5 ml, you will need to transfer the material to a larger tube and add proportionally more K Acetate Solution (1/10 volume) and ethanol (2–2 1/2 volumes). The amount of Glycogen Solution should remain constant, regardless of volume.
- Collect the precipitated mRNA by centrifugation in a microcentrifuge at 4°C for 5 minutes. If the RNA is not to be used immediately, store it in this precipitated state (in ethanol) at -80°C.
- Decant the supernatant and invert the tube over a clean paper towel. Gently tap the tube on the towel to facilitate the removal of excess liquid.
- Redissolve the precipitated RNA in an appropriate volume of Elution Buffer or DEPC-treated water. To determine the "appropriate" volume, consider the RNA concentration desired, the concentration before precipitation ([RNA]), and the volume of the sample subjected to precipitation. Note, however, that the percentage of the RNA recovered after precipitation will depend on the total amount present. With 10 µg of RNA, for example, approximately 70% will be recovered. You may therefore wish to redissolve the pellet in a volume 25-50% smaller than would be required if all of the RNA were recovered.

Appendix 1: Additional Materials

As noted on page 7, all plastic- or glassware which may come into contact with the sample should be autoclaved or otherwise treated to prevent RNase contamination (5).

- *Mechanical or manual tissue homogenizer (optional).*
- *15 ml centrifuge tubes.*
- *1.5 ml screw-top microcentrifuge tubes.*
- *5 ml glass or plastic pipettes.*
- *Centrifuge with swinging-bucket rotor for spun-column chromatography:* Must be capable of accommodating a 15 ml centrifuge tube with a spun column inserted into it.
- *95% ethanol at -20°C.*
- *Spectrophotometer (optional).*
- *0.5 ml quartz glass cuvettes (optional):* Treat by soaking in concentrated HCl: methanol (1:1) for 1 hour and then rinsing several times in DEPC-treated water (see below).
- *DEPC-treated water:* Prepare a 0.1% solution of diethyl pyrocarbonate (DEPC) in distilled water, stir overnight at room temperature, then autoclave (see also ref. 5).

Appendix 2: Second Column Purification

- Invert an Oligo(dT)-Cellulose Spun Column several times to resuspend the matrix.
- Remove both top and bottom closures, place the column in a 15 ml centrifuge tube and balance against a counterweight. Centrifuge at 350 x g for 2 min.
- Remove the column from the centrifuge tube and discard the liquid in the tube. Replace the bottom closure on the drained column and place it upright in a rack.
- Use Elution Buffer to bring the final volume of the RNA sample from Procedure B to 1 ml.
- Incubate the sample at 65°C for 5 minutes then place on ice for 5 minutes.
- Add 0.2 ml of Sample Buffer, and mix gently. Place on ice.
- Prewarm the Elution Buffer to 65°C.
- Apply the sample to the top of the column bed and allow it to enter the matrix under gravity. Remove the bottom closure.
- Balance the column against a counterweight and centrifuge at 350 x g for 2 minutes.
- Apply 0.25 ml of High-Salt Buffer to the column, then centrifuge at 350 x g for 2 minutes. Repeat this wash step with an additional 0.25 ml of High-Salt Buffer, and centrifuge again.
- Using the same centrifugation procedure, wash the column three times with 0.25 ml aliquots of Low-Salt Buffer.

- Place a sterile 1.5 ml screw-top microcentrifuge tube inside a 15 ml centrifuge tube. Place the column into the latter tube with its tip inside the microcentrifuge tube.
- Elute the bound poly(A)$^+$ RNA as follows, using three successive washes with Elution Buffer *prewarmed* to 65°C. For each wash, pipette 0.25 ml of buffer onto the top of the column; balance the column (in its collection tube) against a counterweight; then centrifuge at 350 x g for 2 minutes. Do not change the collection tube between washes, so that the entire 0.75 ml eluate is collected in the same sterile tube.
- Remove the spun column from the centrifuge tube. Using clean (flamed) forceps, recover the microcentrifuge tube containing the column eluate. Place the tube and remaining Elution Buffer on ice.
- Refer to Procedure C for quantitation of the RNA.
- If the RNA is not to be used immediately, the sample should be stored at -80°C as an ethanol precipitate (see Procedure C).

FUNCTION TESTING

Each component of QuickPrep® mRNA Purification Kit is tested to ensure that it is free of RNases. The assembled kit is then tested for its ability to extract calf liver mRNA suitable for direct use in cDNA synthesis.

STORAGE

QuickPrep® mRNA Purification Kit should be stored at 4°C.

ORDERING INFORMATION

QuickPrep® mRNA Purification Kit	27-9254-01

REFERENCES

1. The PCR process is covered by U.S. Patents 4,683,195 and 4,683,202 owned by Hoffmann-La Roche Inc. Use of the PCR process requires a license. Nothing in this publication should be construed as an authorization or implicit license to practice PCR under any patents held by Hoffmann-La Roche Inc.
2. Chirgwin, J. M. *et al.*, *Biochemistry* **18**, 5294 (1979).
3. Pharmacia Biotech, *Analects* 17.4 (1989).
4. Pharmacia Biotech, *Analects* 16.2 (1988).
5. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor page 7.3 (1989).
6. Ausubel, F. M. *et al.*, eds., *Current Protocols in Molecular Biology* 2, 11.5.1 (1989).
7. See reference *5*, page E.5 (1989).

Appendix C

Pharmacia LKB Biotechnology Consumables Catalogue 1992

Chromatography
Electrophoresis
Cell Biology

Pharmacia
Pharmacia LKB Biotechnology

18-1022-51

Oligodeoxynucleotide Celluloses

Pharmacia has developed a variety of oligo(dT)-celluloses for laboratories using molecular biology techniques. Oligo (dT)-cellulose chromatography is the method of choice for mRNA isolation, and also is used for purification of nucleic acid enzymes, binding of steroid receptors and fractionation of oligonucleotides.

Unlike some competitive products, Pharmacia oligo (dT)-cellulose media have a large quantity of medium length (10-20 bases) oligomers on the matrix. This configuration is necessary for successful application of affinity methods. Use the Selection Guide on this page to determine the most appropriate adsorbent for your application.

Features:

- Highly specific for binding poly(A) – isolates poly(A) containing mRNA from 50-100 fold quantities of rRNA
- Can be washed with alkali solutions – allows repeated use of the same column
- Ideal affinity media – Oligo(dT)-cellulose Type 7 contains 40 mg oligo(dt) per gram cellulose with oligo(dT) chains up to 25 nucleotides long

OLIGODEOXYNUCLEOTIDE MEDIA SELECTION GUIDE

Gel	Description	Ligand Substitution	Applications
Oligodeoxynucleotide Media			
Oligo(dT)-Cellulose Type 7	Best oligo(dT)-cellulose available for mRNA isolation. One g is =2.5-3.5 ml. Also available as pre-packed column.	Binds 80-100 A_{260} units poly(A) per g (in 10 mM Tris (pH 7.5) and 500 mM KCl). Binds 40-60 A_{260} units poly(A) per g (in 10 mM Tris (pH 7.5) and 100 mM KCl). Binds >200 A_{260} units poly (A) per g (in 20 mM phosphate (pH 7.5) and 1 M NaCl).	RNA containing poly(A)
Oligo(dT)-Cellulose Type 7F	One g is =2.5-3.5 ml. Contains a higher % of cellulose fines than type 7, resulting in slow flow rates.	Binds up to four times the amount of polyadenylic acid as type 7. Binds 300-400 A_{260} units poly(A) per g (in 10 mM Tris (pH 7.5) and 500 mM KCl).	RNA containing poly(A)
Oligo$(dT)_{12-18}$-Cellulose	Similar to Type 7 and 7A, but contains the longer chain length oligo-nucleotides $(dT)_{12-18}$. One g is =2.5-3.5 ml.	Binds 25-50 A_{260} units poly(A) per g (in 500 mM KCl).	Enzyme purification
Oligo(dA)-Cellulose Type 7	One g is =2.5-3.5 ml.	Binds 25-40 A_{260} units poly(U) per g (in 20 mM phosphate (pH 7.5) and 1 M NaCl).	Poly(U) sequences, such as those which have been shown to occur in Hela nuclear RNA
Oligo(dG)-Cellulose Type 7	One g is =2.5-3.5 ml.	Binds 10-20 A_{260} units poly(C) per g (in 20 mM phosphate (pH 7.5) and 1 M NaCl).	Poly(C) sequences

Type 7. The linkage is through the terminal phosphate of a nucleotide, generally by a phosphodiester bond

ORDERING INFORMATION

PRODUCT	SIZE	CODE NO.	PRODUCT	SIZE	CODE NO.
Oligo(dT)-Cellulose Type 7	500 mg	27-5543-01	Oligo$(dT)_{12-18}$-Cellulose	500 mg	27-5663-01
	1 g	27-5543-02		1 g	27-5663-02
	5 g	27-5543-03	Oligo(dA)-Cellulose Type 7	500 mg	27-5577-01
	Redi-Col*	27-5649-01		1 g	27-5577-02
Oligo(dT)-Cellulose Type 7F	250 mg	27-5671-01	Oligo(dG)-Cellulose Type 7	500 mg	27-5665-01
	1 g	27-5671-02		1 g	27-5665-02

*Redi-Cols are columns pre-packed with 2 ml of affinity media.